Hacettepe University Journal of the Faculty of Pharmacy
Volume 35 / Number 2 / July 2015 / pp. 162-173

Determination of Gelatinase (gelE) Expression Levels of Enterococcus faecalis in Mono and Dual Species Biofilms

Received : 04.11.2015
Revised : 06.01.2015
Accepted : 08.01.2016

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Introduction

Biofilms are known as surface-attached microbial communities and consist of microbial cells and self-produced extracellular polymeric matrix (EPM) including proteins, polysaccharides, nucleic acids and dead cells. Biofilm formation is an important problem threatening human health. They can form in human tissues or attach to the medical devices surfaces. Because of sessile cells in biofilm are highly resistant against antimicrobial agents treating these infections are troublesome[1].

It was shown that in recent studies, most of clinically relevant biofilms leading to chronic infections are polymicrobial. While increasingly being recognized as important, there are few studies on the properties of multispecies biofilms[2]. The dynamics of polymicrobial biofilms are more variable compared to monomicrobial biofilms because of the interactions of different microbial species[3]. The physiology of microbial cells in the biofilm is frequently changed by these interactions and leads to obtain various advantages, such as resistance to antimicrobials or human immune system, metabolic cooperation, quorum sensing systems, more productive gene sharing[4-9].

Enterococci are commensal bacteria of the human gastrointestinal and oral flora. Despite their commensal nature, Enterococcus species have been responsible for many nosocomial infections including bacteremia, central nervous system, urinary tract and surgical wound and endocarditis[10]. *Enterococcus faecalis* isolates lead to most of infections among the other enterococcus species[11]. *Candida albicans* is the fourth most common cause

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of bloodstream infections and can form biofilms on indwelling medical devices[12,13]. It is involved in the highest incidence of mortality[14,15]. *C. albicans* and enterococci have commonly been co-isolated from infection sites and are frequently associated in polymicrobial infections[16,17]. It has previously been shown that the combined effect of *C. albicans* and *E. faecalis* in a mice model results in increasing the growth of enterococci in animals when *C. albicans* had been introduced[18]. In a *Caenorhabditis elegans* model of *E. faecalis* and *C. albicans* mix infection, *E. faecalis* caused the inhibition of *C. albicans* hyphal morphogenesis, a necessary step for *C. albicans* pathogenicity[12].

Differences in the expression levels of some virulence factors of *E. faecalis* have been shown between biofilm producing and non-producing isolates. The gelatinase enzyme, which is an extracellular zinc metalloprotease that hydrolyzes gelatin, casein and collagen, is one of the most important virulence factors of *E. faecalis*[19]. Although there have been many studies on relationship of the biofilm formation and *gelE* expression of *E. faecalis*, it is not clear how the *gelE* expression levels change between mono and dual or polymicrobial biofilms[19-21].

In the present study we investigated the prevalence of biofilm forming ability of 11 *E.faecalis* urine isolates. We also set up an in vitro dual biofilm model in a repeatable style and determined the influence of the presence of *C. albicans* on the *gelE* gene expression levels of a laboratory strain and two isolates of *E.faecalis*.

Material and Methods

Bacterial isolates and growth conditions

A laboratory strain *E. faecalis* ATCC 29212 and eleven *E. faecalis* clinical isolates from urine samples of patients in intensive care units were used for monomicrobial biofilms. These isolates were maintained as frozen stocks at -80°C. Before using, all isolates were subcultured onto tryptone soya agar (TSA) (Merck, Darmstadt, Germany), and then cultured overnight in tryptone soya broth (TSB) (Merck, Darmstadt, Germany) at 37°C. Bacterial cells were washed in phosphate-buffered saline (PBS) (Medicago AB, Uppsala, Sweden) by centrifugation at 4000 rpm for 15 min and final inoculum suspensions of all clinical strains were adjusted to approximately 10^6 CFU ml^{-1}. Each experiment included the sterile TSB as negative control and the biofilm-forming *E. faecalis* ATCC 29212 strain as positive control. For dual biofilm formation; an *E.faecalis* ATCC 29212 laboratory strain and two *E. faecalis* isolates were cultured with *C.albicans* ATCC 90018 separately (10^6 cfu/ml, 10^5 cfu/ml in brain heart infusion broth (BHI) (Merck, Darmstadt, Germany) for the final inoculum concentration of bacteria and yeast respectively).

Gelatinase activity assay

Gelatinase production was screened phenotypically by inoculating single colonies of all twelve E.faecalis onto Todd Hewittt agar plates (Fluka Analytical) containing 3% gelatin. After incubating the plates at 37^{0}C for 24 h, they cooled for 5 h at 4 ^{0}C. Appearance of turbid zones around the colonies was determined as gelatinase production[22].

Biofilm formation assay

For monospecies biofilms, 100 µl of the final inoculum suspension of 11 *E. faecalis* isolates (10^6 cfu/ml) and the laboratory strain of *E. faecalis* (10^6 cfu/ml) were added to the 12 wells of a flat-bottomed polystyrene 96 well microtiter plate separately. For dual species biofilms, *E. faecalis* isolates were co-cultured with a laboratory strain of *C. albicans* (10^5 cfu/ml) and incubated at 37°C without shaking. Sterile BHI was used as the blank. After 4 hours, the wells were washed with 100 µl of sterile PBS, then 100 µl of fresh BHI was added and the plates were incubated for an additional 20 hours for mature biofilm formation. After 24 hours, supernatants were removed and each well was washed with PBS before quantifying the biofilm cells[2].

Biofilm quantification

CV staining

In order to quantify the biomass of *E. faecalis* biofilms, we used an optimized assay[23]. Briefly, wells were washed three times with sterile PBS then the plates were air-dried. The wells were stained with 0.2% crystal violet for 15 min and repeatedly washed three times with PBS to remove excess dye. The plates were dried for 15 min at room temperature and the bound crystal violet was solubilized in 150 µl of acetone/ethanol solution. The optical densities (ODs) of the stained cells were read at 590 nm on a micro-ELISA plate reader. We defined the cut-off OD (0.282) as three standard deviations above mean OD of the negative control. We categorized our strains as:

OD $\leq$ Cut-off OD (0.282) as a negative biofilm producer,

Cut-off OD (0.282) < OD $\leq$ 2×Cut-off OD (0.564) as a weak biofilm producer,

2×Cut-off OD (0.564) < OD $\leq$ 4×Cut-off OD (1.128) as a moderate biofilm producer,

OD > 4×Cut-off OD (1.128) as a strong biofilm producer.

Plate counting

Plate counting assay was used for the quantification of the number of cells in monomicrobial and polymicrobial biofilms. Both biofilm formations were detached by vortexing (5 minutes) followed by sonication (5 minutes). This procedure was repeated after adding 100 µl of PBS to each well, and sonication fluids were transferred to the sterile tubes. Serial dilutions were made in sterile PBS and plated onto TSA for *E. faecalis* monomicrobial biofilms. The number of sessile cells of *E. faecalis* and *C. albicans* in dual biofilms were determined by plating the dilution solutions onto the TSA supplemented with 4 mg/ml amphotericin B (Sigma, Life Science) and SDA (Merck, Darmstadt, Germany) including 16 mg/ml vancomycin (Sigma, Life Science), respectively After 24 hours, the number of CFU $ml^{-1}$of the colonies were counted.

Expression of the *gelE* gene in mono and dual species biofilm cells of *E. faecalis*

Total RNA was extracted from mono and dual-species biofilm cells of *E. faecalis* ATCC 29212 and *C. albicans* ATCC 90018 with the RNeasy® Mini Kit according to the manufacturer's recommendations (Qiagen GmbH, D-40724 Hilden, Germany). The RNA concentrations were determined by a Nanodrop ND-1000 apparatus. All RNA extracts were prepared as 100 ng μl^{-1} per sample and transcribed into cDNA using Transcriptor High Fidelity cDNA Synthesis Kit according to the manufacturer's instructions (Roche Diagnostics GmbH, Manheim, Germany). Real-time PCR (Roche Light Cycler 2.0) was performed with LightCycler Faststart DNA Master SYBR Green1 (Roche Diagnostics GmbH) in a total volume of 20 µl. Primer sequences for the housekeeping gene *16sRNA* and *gelE* were obtained from the literature[24]. The*16sRNA* gene was used to normalize the expression level of *gelE*. Melt curve analysis was carried out to assess the specificity of each primer pair. The comparative C_T method for relative quantification ($^{\Delta\Delta}C_T$ method) was performed to analyze the data[25]. The threshold cycle (C_T) is the point at which the level of fluorescence starts to exponentially increase above background fluorescence and is used to measure the gene expression level. The fold changes in expression of *gelE* gene relative to the *16sRNA* gene in mono-species and dual-species biofilm forms were determined. Three independent experiments were performed.

Statistical analysis

All experiments were repeated three times. Statistical analyses were performed using SPSS software, version 21. The results of gene expression experiments were analyzed by t-tests and only differences of more than two fold up- or downregulation and with a *P*-value < 0.05 were considered significant.

Results and Discussion

All of the *E. faecalis* strains were found as biofilm producer by both CV and plate counting methods. One half of the isolates were detected as weak biofilm producer and the other half were moderate with CV assay. Minimum logaritmic cfu/ml value was found as 8 with plating assay which means that minimum 2 log increase was observed when we compared with the final starting inoculum (6 log cfu/ml). It shows that all the isolates attached to the wells of the plates and grow to form mature biofilm at 24 hours.

We carried out the phenotypic assay for the gelatinase activity of 12 *E. faecalis* planktonic forms and found only 2 isolates with positive gelatinase activity. Interestingly, we detected an increased gelatinase gene expression in the biofilm cells of *E. feacalis* by RT-PCR (Figure 2). Some of the virulence factor genes such as gelE were found to be involved in biofilm formation of *E.faecalis* and an increased expression level of the gene has been shown in biofilm producing isolates compared to non-producing isolates[34]. Our results of gelatinase activity in planktonic and biofilm forms of *E.faecalis* are also consistent with the literature.

TABLE I

Biofilm forming ability and phenotypic gelatinase activity results of 11 *Enterococcus faecalis* isolates and the laboratory strain.

Isolate No	*OD_{590nm}	Biofilm Category	§Log cfu/ml	Gelatinase Activity
1	0.33	weak	8.8	+
2	0.40	weak	8	-
3	0.43	weak	9.5	-
4	0.50	weak	9.4	-
5	0.62	weak	10.3	-
6	0.69	weak	9.3	-
7	0.86	moderate	9.9	-
8	0.98	moderate	8.8	-
9	1.02	moderate	8.5	+
10	1.03	moderate	9.5	-
11	1.06	moderate	10.3	-
12[a]	1.06	moderate	9	-

*: Mean optic density value of *E.faecalis* isolates and ATCC strain by CV assay (Cutoff OD: 0.282) §: Mean logaritmic cfu/ml value of *E.faecalis* isolates and ATCC strain by plating assay. a: *E.faecalis* ATCC 29212, Results are means of at least 3 different experiments.

We tested whether *E. faecalis* and *C. albicans* could form a dual-species biofilm in the microtiter plate and observed bacterial and fungal growth within the biofilms by the plating assay using selective agar plates to assess fungal versus bacterial counts. Logaritmic cfu per ml of *E. faecalis* remained the same in mono and dual species biofilms (Fig. 1). However, *C. albicans* levels were significantly decreased in the dual species biofilms formed by *C. albicans* and *E. faecalis* compared to *E. faecalis* monomicrobial growth (Fig. 1). These data demonstrated that *C. albicans* and *E.faecalis* formed a polymicrobial biofilm and the number of *C. albicans* cells was influenced negatively in the presence of *E. faecalis.*

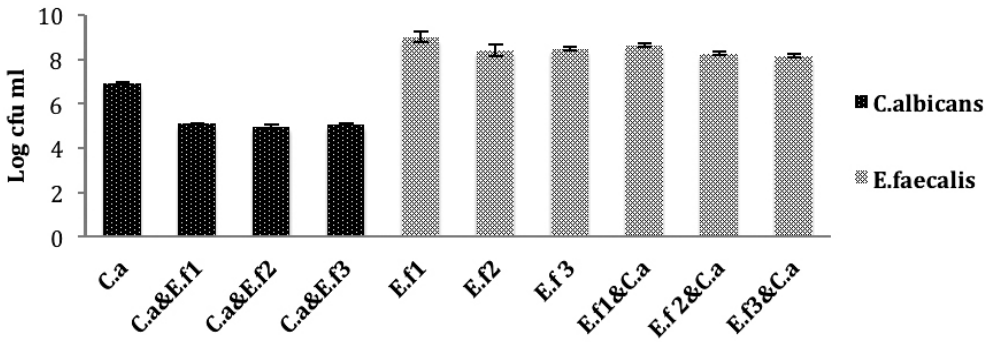

Figure 1

Sessile cell counts of *C. albicans* and *E. faecalis* in mono and dual biofilms C.a and E.f represents logarithmic cell counts in monomicrobial biofilms of *C. albicans* and *E. faecalis*, respectively. C.a&Ef1, C.a&E.f2 and C.a&E.f3 (black bars) represents logarithmic cell counts of *C. albicans* in dual biofilms with three different *E.faecalis* clinical isolates. E.f1&C.a, E.f2&C.a and Ef3&C.a (gray bars) represents logarithmic cell counts of three clinical Enterococcus isolates in dual biofilms with *C. albicans,* respectively. The data represent the averages of three experiments. Values which were significantly different ($p \leq 0.05$, Mann whitney test) for the *C. albicans* mono-species biofilm versus the *C. albicans* dual-species biofilms are indicated by the asterisks.

It has previously been demonstrated that one species can have a negative or positive effect on the growth or antimicrobial sensivity profiles of another species. A dual species biofilm of *C. albicans* and *Staphylococcus epidermidis* increases the growth of *S. epidermidis* and also resistance of *S. epidermidis* to vancomycin[26,27]. In a *P. aeruginosa* and *C. albicans* mix biofilm models, it was observed that *P. aeruginosa* formed biofilms on the fungal filaments of *C. albicans* and this close contact caused the killing of the fungal filaments[28-31]. Another study showed the altered antimicrobial sensitivity in the mix bio-

films of *C. albicans* and *S. aureus*. Although *C. albicans* showed no difference in sensitivity to amphotericin B, *S. aureus* resistance to vancomycin was enhanced within the dual biofilm.

We also compared the *gelE* mRNA levels of sessile cells of *E. faecalis* ATCC 29212 and two isolates in mono-species biofilms with dual-species biofilm formed with *C. albicans* ATCC 90018. Our results showed that the *gelE* expression levels of *E. faecalis* ATCC 29212 and the isolates were significantly enhanced in the dual species biofilms of *E.faecalis* and *C.albicans* when compared to their mono-species forms ($p \leq 0.05$). Increases in biofilm formation of *E.faecalis* biofilm by *gelE* were shown in some studies[32, 33]. Although there are a great number of studies on relationship of biofilm formation and *gelE* expression in planktonic and monomicrobial biofilm forms of *E. faecalis*, it has not yet been investigated the mRNA levels of *gelE* between in mono and dual-species forms[34-40]. To our knowledge, it is the first study showing the positive effect of *C. albicans* on the *gelE* gene expression of *E. faecalis* in the biofilms found together. Further studies with clinical strains will be needed to support this increased *gelE* expression results in the dual biofilm.

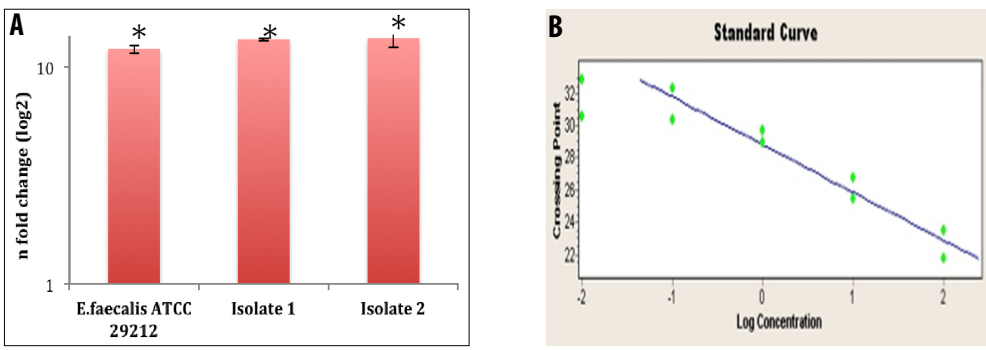

Figure 2

GelE gene relative expression ratio for *E.faecalis* ATCC 29212 and two clinical isolates. Bar values reflect fold changes in gelatinase mRNA levels of *E.faecalis* bacteria between mono and dual biofilms with *C.albicans*. *: $p \leq 0.05$, Mann whitney test

Conclusion

All *E. faecalis* strains were found as biofilm producer by both quantification methods.

Minimum 1 log reduction in *C. albicans* cell numbers were observed when grown in dual biofilms of *C. albicans* and *E. faecalis. E. faecalis* showed inhibitory effect on *C. albicans* growth in biofilms. GelE expressions were significantly higher in dual biofilms than mono-species biofilms of *E. faecalis* strains ($p \leq 0.05$).

Abstract

Biofilms are substantial problem threatening human health. Recently, it was shown that most of biofilms leading to persistent infections were polymicrobial. *E. faecalis* is an opportunistic pathogen frequently isolated from hospital infections such as urinary tract, wound infections etc. Differences on expression levels of some virulence factors of *E.faecalis* were determined between biofilm producing and nonproducing isolates. Gelatinase, a metallo protease enzyme hydrolyzing gelatin, collagen and caseine, is one of the virulence factors of *E.faecalis*. The role of gelatinase with biofilm formation was found in some studies using mutant strains. Our first aim is to determine the prevalence of biofilm forming ability of 11 *E.faecalis* urine isolates. Our second aim is to set up an in vitro dual biofilm model in a repeatable style and to find out the impact of the presence of *C.albicans* on the *gelE* gene expression of *E.faecalis* by using RT-qPCR. In the present study, all isolates were found as biofilm producer by both method. Moreover, our results showed increased *gelE* expression in dual species biofilm formed by *C. albicans* ATCC 90028 and *E. faecalis* ATCC 29212 when we compared with *E. faecalis* monomicrobial biofilm form. Further studies with clinical isolates will be needed to support this increased *gelE* expression level in the dual biofilm.

Keywords: Biofilm, E.faecalis, Gelatinase gene, Polymicrobial, Expression

Özet

Tek ve iki türlü biyofimlerde *E.faecalis* jelatinaz gen ekspresyon seviyelerinin belirlenmesi

Biyofilmler insan hayatını tehdit eden önemli bir problemdir. Son yıllarda, persistan enfeksiyonlara yol açan biyofilmlerin çoğunun polimikrobiyal olduğu gösterilmiştir. *Enterococcus faecalis* idrar yolu, yara enfeksiyonları vb.

gibi hastane enfeksiyonlarından sıklıkla izole edilen fırsatçı bir patojendir. *E.faecalis*'in bazı virulans faktörlerinin ekspresyon seviyelerinde, biyofilm oluşturabilen ve oluşturamayan izolatları arasında farklılıklar gözlenmiştir. Jelatinaz, jelatini, kollajeni ve kazeini hidrolize eden metallo-proteaz enzimi olup, *E.faecalis*'in virulans faktörlerinden birisidir. Mutant suşlar kullanılarak yapılan bazı çalışmalarda bu enzimin biyofilm oluşumundaki rolü bulunmuştur. İlk amacımız, 11 *E.faecalis* idrar izolatının biyofilm oluşturabilme prevalansını belirlemektir. İkinci amacımız, tekrar edilebilirliği yüksek iki farklı mikrobiyal türden oluşan biyofilm modelini in vitro ortamda geliştirmek ve C.albicans'ın ortamdaki varlığının *E.faecalis*'in gelE gen ekspresyonu üzerindeki etkisinin kantitatif RT-PCR ile belirleyebilmektir. Çalışmamızda, test edilen her iki yöntemde de tüm izolatların biyofilm oluşturabildiği saptanmıştır. Ayrıca, *E.faecalis* ATCC 29212 suşu tarafından oluşturulan monomikrobiyal biyofilm modeline oranla, C.albicans ATCC 90028 ve E.faecalis ATCC 29212 tarafından oluşturulan ikili biyofilm modelinde gelE ekspresyon artışı olduğu sonuçlarımızda gösterilmiştir. İki türden oluşan biyofilm modelinde gözlenen bu gelE ekspresyon seviyesindeki artışı desteklemek amacıyla klinik izolatlar kullanılarak yapılacak çalışmalara ihtiyaç duyulmaktadır.

Anahtar kelimeler: Biyofilm, E.faecalis, Jelatinaz geni, Polimikrobiyal, Ekspresyon

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